FINE STRUCTURES OF THE CARBON MONOXIDE SECRETING TISSUE IN THE FLOAT OF PORTUGUESE MAN-OF-WAR PHYSALIA PHYSALIS L.) [1]

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The Portuguese-man-of-war has been sighted in all seas of the world and has intrigued naturalists for hundreds of years. Much is known about its morphology and general behavior. These aspects have been well summarized in the companion monographs of Totton (1960) and Mackie (1960). Totton carefully outlines the development of the animal which starts as a single hydranth-like form (with one tentacle and a small float) and progresses to a very complex form with modified hydranths budded from but still attached to the original body. He also includes interesting observations on right-hand sailing and left-hand sailing by individuals. His interpretations of the possible significance of the phenomena are at variance with those of Woodcock (1944, 1956).

Despite the long-term interest in *Physalia* very little is known of its physiology. This may be due to the fact that the animal is extremely sensitive to confinement. If placed in an aquarium, it will start degenerating in a day or two. Charles E. Lane, who has made many observations of *Physalia* on the Florida coast off the Institute of Marine Sciences, University of Miami, believes that if the extended tentacles repeatedly touch solid bottom, the animal is adversely affected (personal communication). He feels that a cylindrical sea water tank 50 feet tall, 20 feet wide and with air jets about the periphery of the top to keep the animal centered would probably solve the problem of survival in captivity.

Observations have been made on the gas content of the float by previous workers such as Schloesing and Richard (1896). It remained for Wittenberg (1958, 1960) to first describe significant ratios of carbon dioxide in the gases, ranging up to 8% of the total. This observation has been further explored by Clark and Lane (1961), Wittenberg, Noronha and Silverman (1962), Larimer and Ashby (1962) and Hahn and Copeland (1966).

Another colonial siphonophore, the bathypelagic *Nanomia bijuga* recovered from the deep scattering layers off the California coast, has been reported to possess as much as 90% carbon monoxide in its floats (Pickwell, Barham and Wilton, 1964). This form should receive further attention.

Another instance of carbon monoxide production is in the case of the Pacific bladder kelp (*Nereocystis luetkeana*). This is a kelp that may reach 85 feet in length and have up to 4 liters of gas in the stipe and bulb. Langdon (1917) reports the normal occurrence of an average of 4% (range 1 to 12) carbon monoxide in

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the gas. The gas cavity is sterile and therefore it was concluded that the monoxide gas is produced by the plant in its normal respiration (Rigg and Henry, 1935).

There are many references to carbon monoxide production in other forms but these deal with special circumstances. Metz and Sjöstrand (1954) recorded small amounts of the gas released by guinea pigs and rabbits, probably accounted for by the decomposition of hemoglobin. Wilks (1959) by grinding up alfalfa and putting it in a flask exposed to direct sunlight obtained traces of the gas. Westlake *et al.* (1961) by exposing moulds to various substrates were able to show that the metabolic pathway, under certain circumstances, may use 1 carbon metabolites. Siegel *et al.* (1962) found that seedlings under reduced oxygen tension (5% or less) could produce measurable amounts of carbon monoxide. Loewus and Delwiche (1963) found no carbon monoxide in the floats of the brown alga *Egregia*

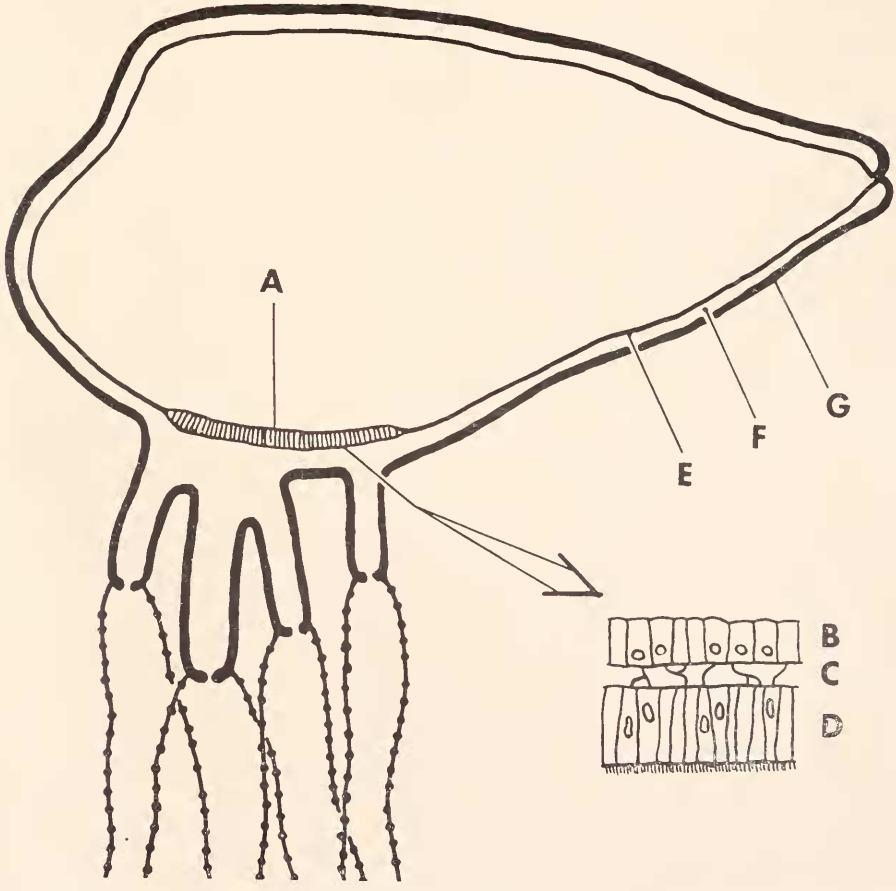


FIGURE 1. Diagram (schematic) of *Physalia* to depict relationship of the gas gland (pneumadena) to the rest of the body. A. The pneumadena (gas gland). B. Ectoderm of pneumadena. C. Mesoglea. D. Gastroderm. E. Pneumatosaccus. F. Gastrovascular cavity. G. Pneumatocodon.

menzies but if they homogenized the alga, carbon monoxide could be produced by the dissociated tissues.

It would therefore appear that the siphonophores *Physalia* and *Nenomia* and the bladder kelp *Nerocystis* are the only forms known to date that produce large amounts of carbon monoxide as part of their normal physiology.

The gas is secreted in *Physalia* by a disc of tissue (pneumadena) on the ventral lining surface of the float (Fig. 1). Again, surprisingly little is known of the histology of the gas-secreting tissue. Incomplete descriptions have been given by Okada (1935) and by Mackie (1960). A description by Dahlgren and Kepner (1908) is probably erroneous.

No description of fine structure observed with the electron microscope has been reported except my own preliminary notes (Copeland, 1962, 1966). This report now presents my morphological observations to date.

Materials and Methods

Initial observations were made on *Physalia* collected in the open Atlantic off Gay Head Light, Martha's Vineyard, Massachusetts, in August, 1961. A week of prevailing southerly winds drifted the animals into the area from the Gulf Stream in considerable numbers. Although they appeared normal, it was subsequently discovered that they were in a degenerating condition compared with forms collected in the Gulf of Mexico off the Mississippi River Delta.

Investigations of the physiology of carbon monoxide secretion (Hahn and Copeland, 1966) indicated that the gas-secreting system of *Physalia* is quite sensitive to lowered temperatures and, probably, to physical handling. Therefore, fixation was done aboard boat immediately after collection. The best collecting area was 30 to 50 miles off South Pass, beyond the brown to green Mississippi River fresh water overlay and in the blue, open Gulf water. Gas samples were analyzed for each animal (method, Hahn and Copeland, 1966). Tissues from animals with less than 10% carbon monoxide float gas content were discarded. Concentrations as high as 25–28% were not unusual. The highest recorded concentration was 35% in one individual.

As soon as an animal was netted and the streaming tentacles cut off it was placed on a wire gauze frame in a deep ice chest. The entire float of the animal was thus exposed to ice cold air. After 5 to 10 minutes of cooling, dissection was commenced.

The gas-secreting epithelium is a single cell layer in thickness and easily disrupted by direct injection of fixative into the float and on to the surface of the gland. An osmic fume fixation procedure, as used in the study of gas secretion in the teleost swim bladder (Copeland, 1968), was of some aid but the best fixation procedure involved use of 5% glutaraldehyde (Sabatini *et al.*, 1963) buffered to pH 7.4 with S-collidine buffer (Bennett and Luft, 1959). This was followed by post-fixation in 1% osmic acid in the same buffer. Both fixatives and the intermediate buffer rinse were brought to 950 milliosmoles by the addition of sucrose (Caulfield, 1957).

By use of curved, fine-pointed scissors the pneumatocodon was slit for the length of the animal and the intact inner bladder (pneumatosaccus) was rolled out. The still inflated pneumatosaccus was held with the gas gland (pneumadena)

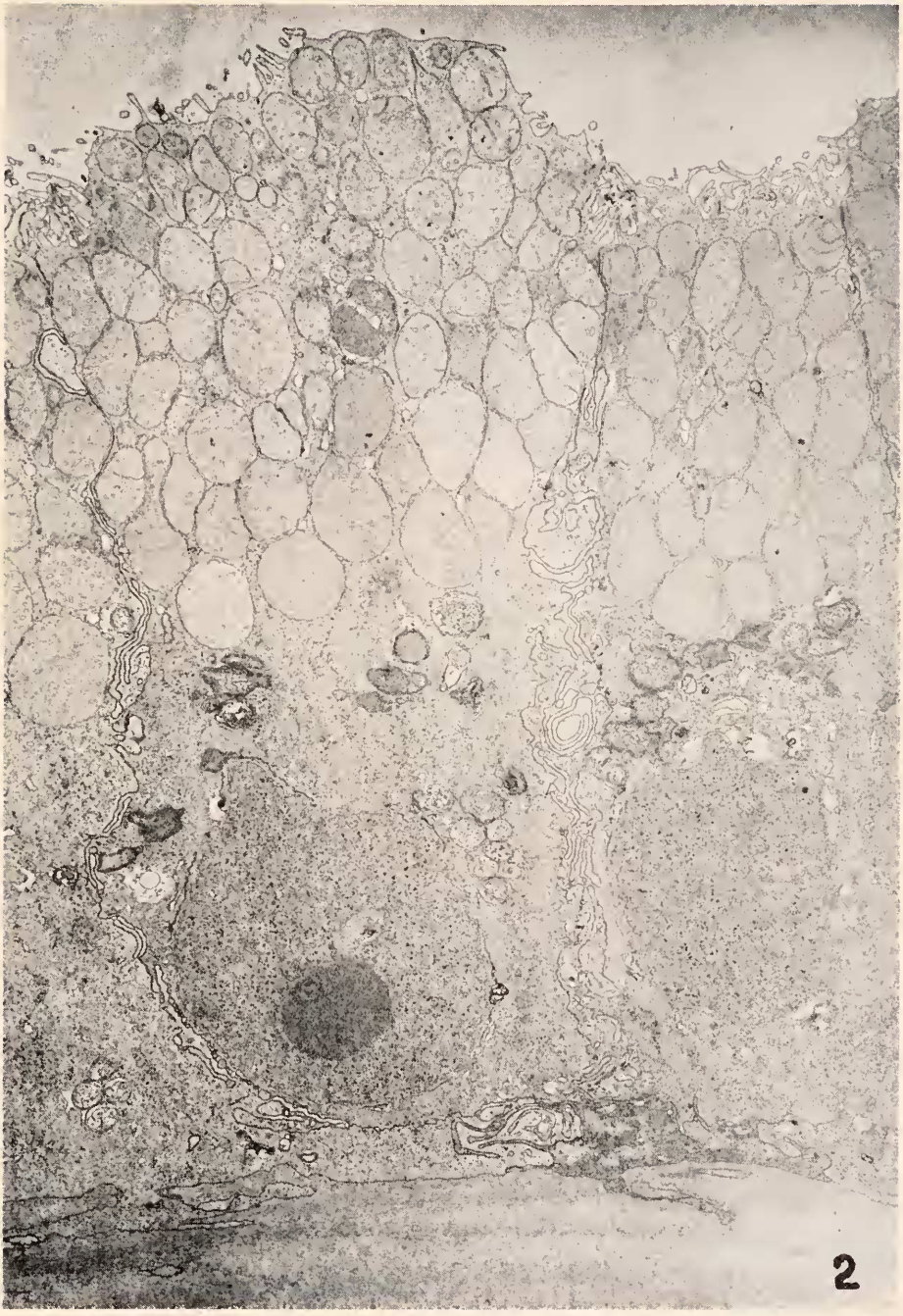
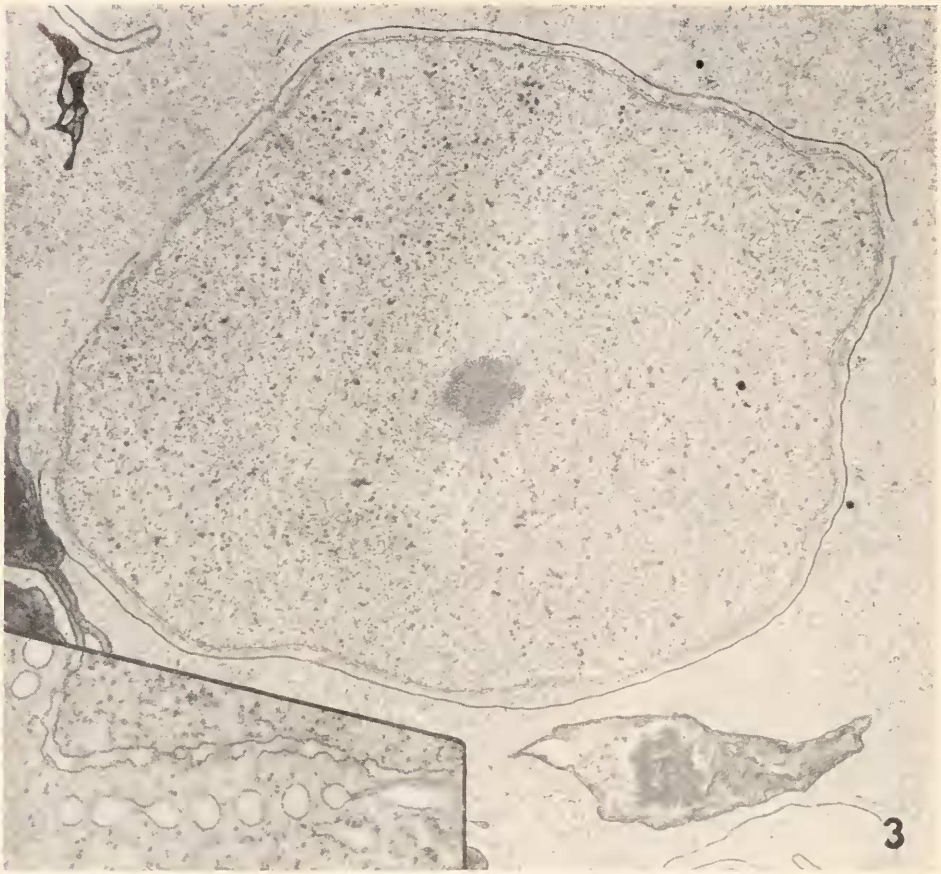
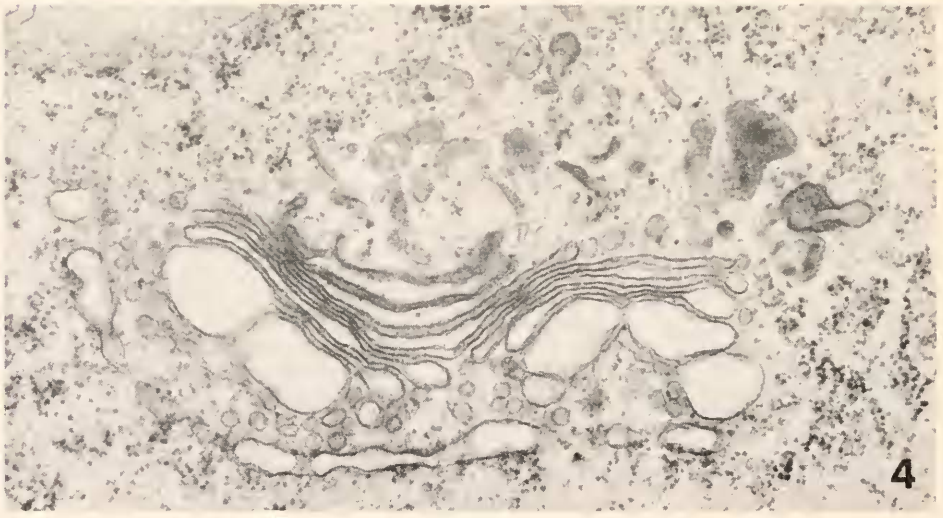


FIGURE 2. Whole cell of pneumadena layer. Gas interface is at top and mesogleal bounding layer is at bottom. See text and other figures for amplification. 4,500 ×.





FIGURES 3–4.

downward and against the surface of the chilled glutaraldehyde fixative. The relatively thick gastroderm and mesoglea were traversed by the fixative before it reached the gas-secreting ectodermal layer. Thus the delicate gas interface surface of the pneumadena was not disturbed by direct contact and the insulating effect of the gastroderm and mesoglea also resulted in more consistent fixation of the ectodermal cells throughout their depth. After 10 minutes of preliminary exposure, some of the same fixative was injected into the cavity of the pneumatosaccus, flooding the ectodermal surface of the pneumadena. After another 10 minutes the pneumatosaccus was lifted from the surface of the fixative, collapsed on dental bite-wax, the pneumadena cut free with a razor blade and placed in a vial to complete a total of 3 hours fixation with glutaraldehyde. Then followed repeated changes of buffer rinse for approximately 2 hours. Post-fixation in 1% osmic acid was for 1 hour. Dehydration was rapid, starting with 50% ethanol and proceeding with constant agitation to absolute in less than an hour. Tissues were then brought to room temperature for final trimming and embedded in Epon 812 by the method of Luft (1961). Thin sections were double stained with uranyl acetate (Watson, 1958) and lead citrate (Reynolds, 1963).

Results

The ectodermal layer of the pneumadena is composed of a single layer of columnar cells with basally located nuclei and large, closely packed mitochondria in the distal halves (Fig. 2). The basal surfaces of the cells frequently have extensions that continue into the mesoglea and interdigitate with similar extensions protruding from the ends of the gastroderm cells.

The nuclei are irregular but tend to be spherical or slightly lobate in contour. In the Gay Head Light material the nuclei were frequently surrounded by an even row of vesicles (Copeland, 1962). In the more healthy material of the Gulf of Mexico vesicles were occasionally seen but they were considered to be due to breakdown of a cisternal space frequently seen adjacent and parallel to the nuclear membrane. The cisternal space itself is not always visible but the membranes are quite evident (Fig. 3).

Only scanty endoplasmic reticulum is seen. It is usually of the smooth variety and randomly disposed. A branch of the endoplasmic reticulum is to be observed in association with the Golgi complex when that structure is cut at right angles to its cisternal spaces (Figs. 4 and 5).

The Golgi complexes are usually found just below the mitochondrial zone and have a characteristic configuration (Fig. 4). A flattened unit of the endoplasmic reticulum apparently delivers small vesicles that coalesce into the large cisternal spaces of the Golgi apparatus. The latter then condense and finally release dark, formed bodies at the delivery side of the complex.

A wide range of tinctorial values are to be seen in the cells. Figure 5 illustrates a case of marked contrast. There seems to be no other significant

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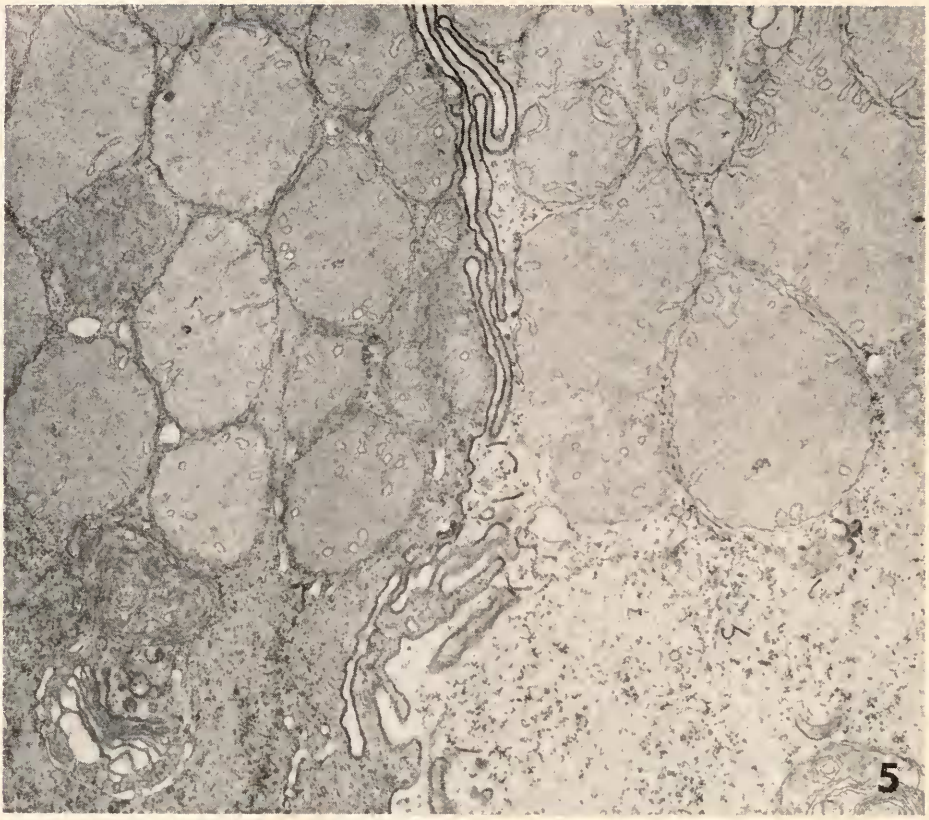
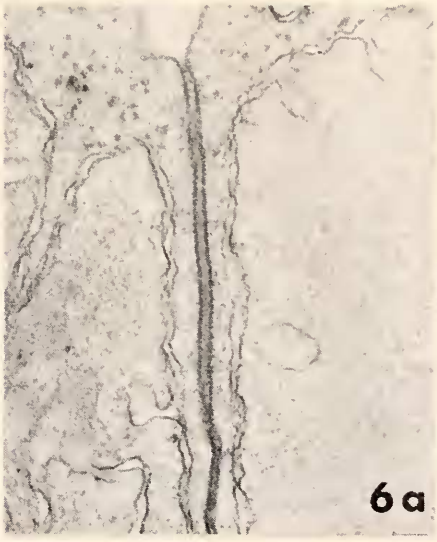
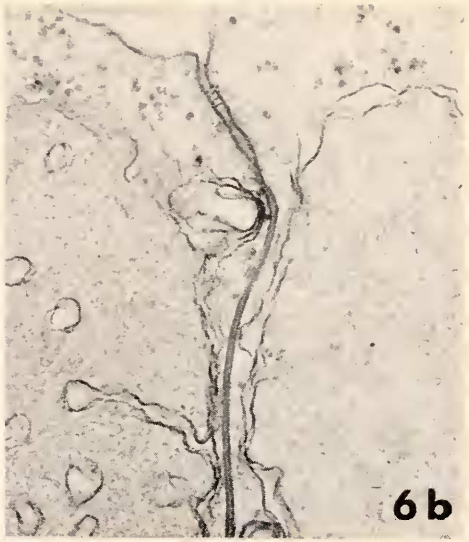
Figure 3. Nucleus surrounded by cisternal membranes. Cisternal space has collapsed. Inset shows cisternal space broken into row of vesicles (Gay Head Light, Massachusetts, material). 12,000 ×.

Figure 4. Golgi complex. Endoplasmic reticulum space at bottom. Delivery side of complex is toward top. 40,000 ×.







FIGURES 5–6.

difference between such cells except in the case of extremely dark, obviously moribund cells. Whatever the reason for the tinctorial difference it must be a general one because the entire cell is involved. Figure 5 also well illustrates the mild degree of overlapping interdigitation between the adjoining sides of neighboring cells.

The junction between neighboring cells is plain except for a density between the plasma membranes at the distal ends of the cells near the gas interface (Fig. 6a). Under suitable orientation of sectioning, at least part of the density is seen as a typical septate desmosome (Fig. 6b). Typical zonulae occludentes, zonulae adhaerentes or maculae adhaerentes are not seen.

Immediately below the zone of closely packed mitochondria and sometimes extending down each side of the nucleus is a zone populated by multivesiculate bodies (Fig. 7). These are numerous, usually have multiple encapsulating membranes and internal vesicles that are spherical or oval, sometimes tubular in shape. The multivesicular bodies seem to arise by breakdown of encapsulated mitochondria that become isolated from those above. The initial encapsulation may also trap some of the adjacent cytoplasm (Fig. 7). However, the exact origin and fate of the multivesiculate bodies is not clear at this time.

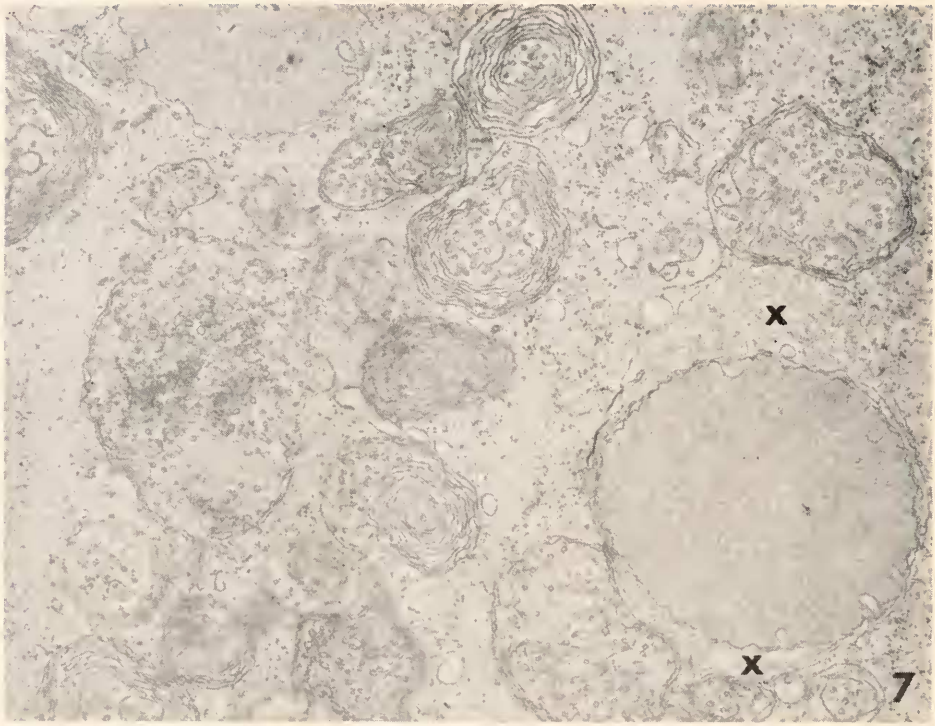
The mitochondria are quite noteworthy. They are oval in form and closely packed in the distal part of the secretory cell (Fig. 8). They differ from most types of mitochondria in having very few cristae. These are short and tubular in the main, but they occasionally form flattened extensions from one side back to the same side or across to the opposite membrane (Fig. 8). Almost the total bulk of the mitochondrion is occupied by a dense granular matrix. Irregular, dark particles are occasionally found in the granular matrix.

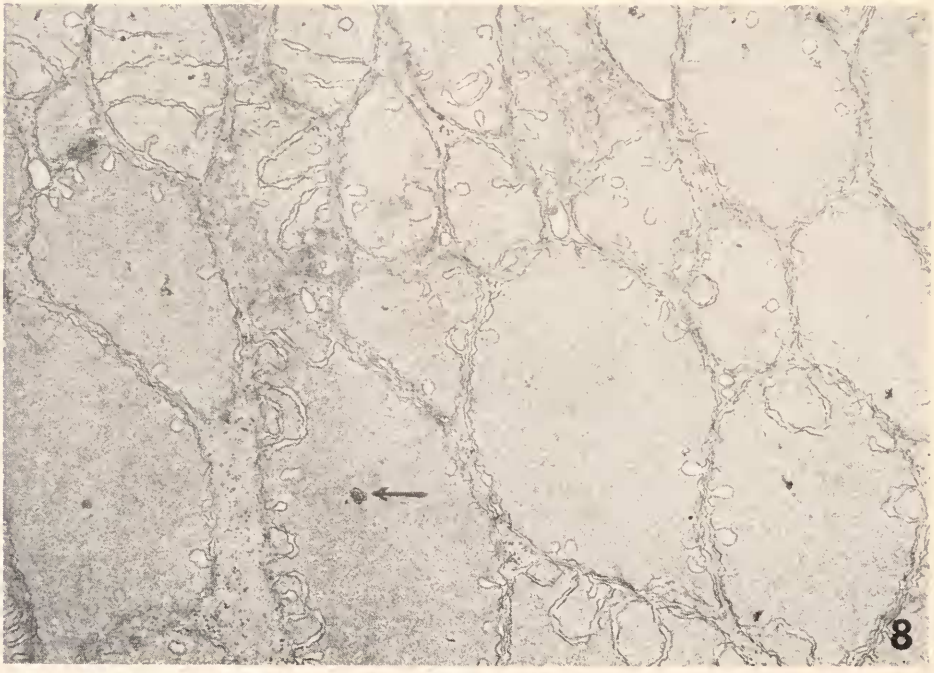
In well fixed material the gas surface interface of the secretory cells is seen to have many irregular projections (Fig. 9). These are not regular enough to be called microvilli but there is a resemblance. A few vacuoles or vesicles are seen near the surface but not in sufficient numbers to warrant the certainty that they are occupied with secretion of free gas. Many of them are probably oblique sections of the crypts formed by the bases of the cytoplasmic surface projections.

In a preliminary report (Copeland, 1962) it was stated that the distal end of the secretory cells possessed numerous rows of vesicles possibly devoted to gas release and that the cell was devoid of mitochondria. It was hypothesized that in the presence of high carbon monoxide levels the ectodermal cells had become reliant on respiratory and metabolic support from the gastroderm cells *via* the cytoplasmic bridges across the mesogleal layer. This hypothesis may still have some merit but the interpretation was based on a degenerating condition. The 1962 report was based on material collected in the relatively cold waters off Gay Head Light, Massachusetts. Figure 10 illustrates the morphology characteristic of that material. Figure 11 illustrates an intermediate condition observed in material collected in the Gulf of Mexico which had not degenerated as much.

FIGURE 5. Junction of a light and dark cell. Golgi complex at extreme lower left. Both cells appear normal. Interdigitation clearly shown. 12,000 ×.

FIGURE 6. Cell junctions, seen as parallel plasma membranes with dark substance between them (a). Occasionally the dark substance appears septate (b). Both: 55,000 ×.





FIGURES 7–8.

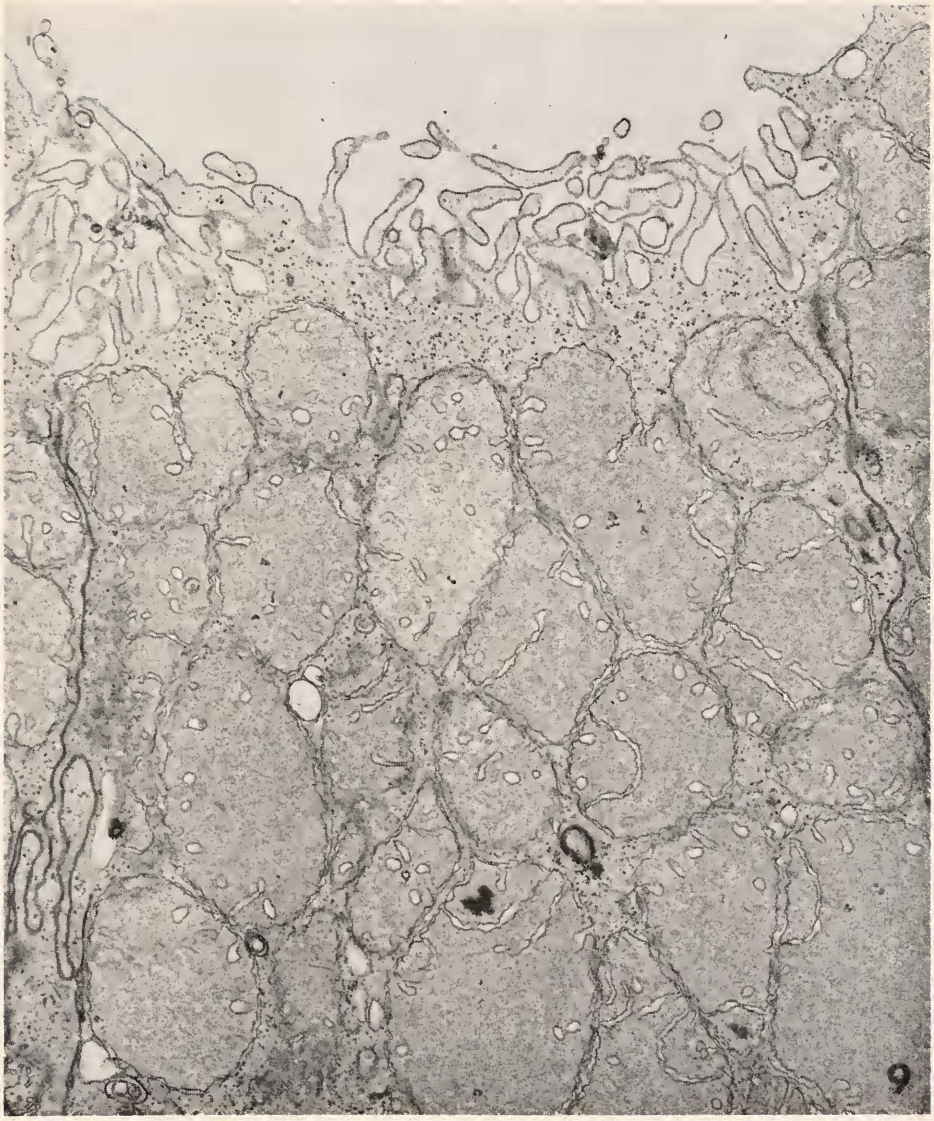
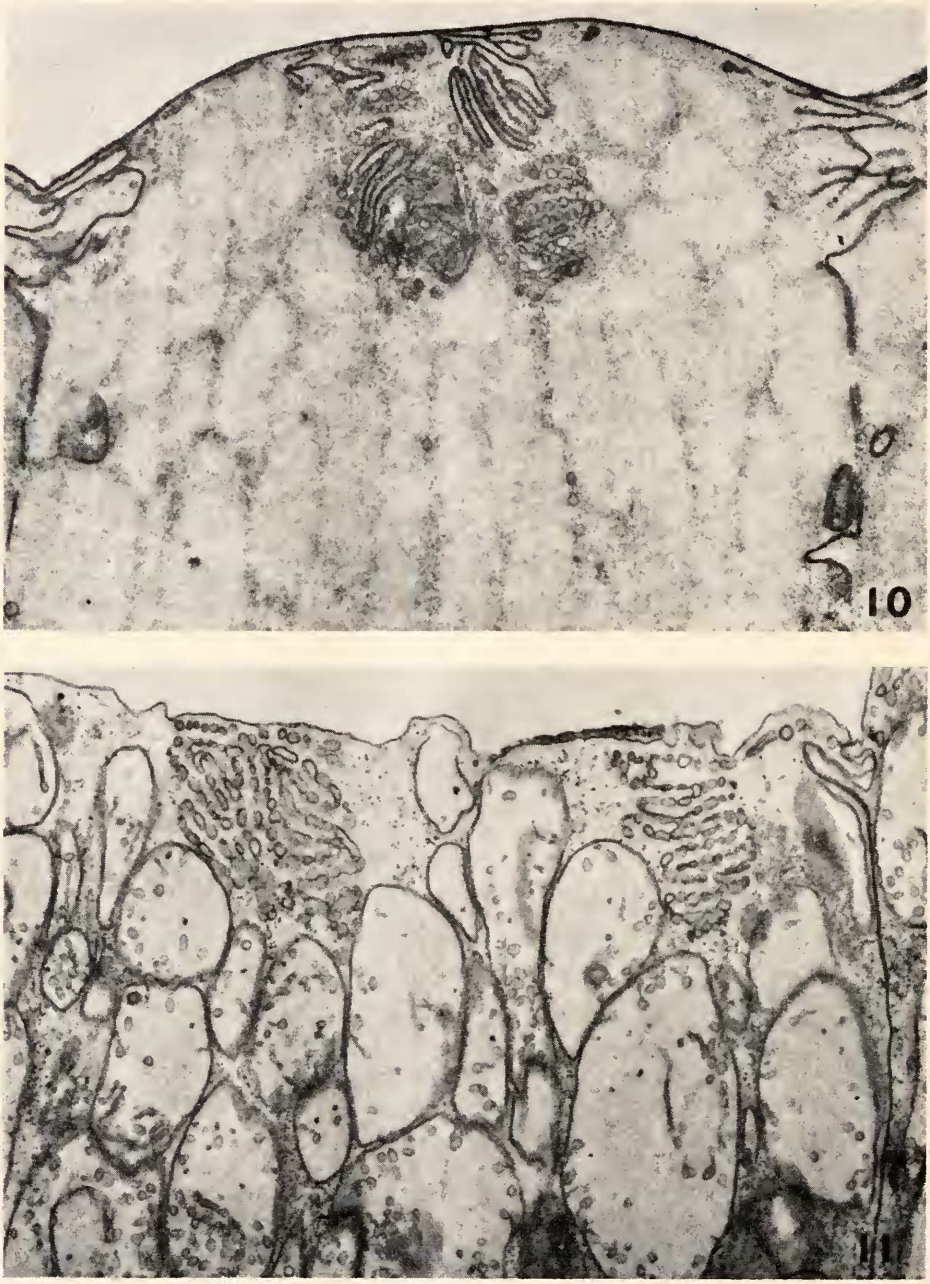


FIGURE 9. Gas surface interface. Note the many irregular projections of the surface. 18,000 ×

Figure 9 is a comparable view in which no degeneration is visible. It and all the illustrations except Figure 10 are of material collected off the Mississippi River Delta area.

FIGURE 7. Multivesiculate bodies of varying complexity. Edge of normal mitochondria seen above. To right is an encapsulated mitochondrion with some cytoplasm (X's) included in the capsule. 19,000 ×.

FIGURE 8. Mitochondria in detail. Cristae quite sparse and short. They are mainly tubular, though long flattened ones are occasionally seen. Occasional, irregular dark bodies are seen in the matrix (arrow). 18,000 ×.



FIGURES 10–11.

FIGURE 10. "Degenerate" cell type observed in the material fixed off Gay Head Light, Massachusetts. Mitochondria completely fragmented. "Gas release vesicles" seen just below surface of cell. (Compare with Figures 9 and 11.) 11,500 ×.

As a final observation, it should be stated that the external appearance of the *Physalia* is not a reliable indication of the physiological condition of the pneumadena. The relative percentage of carbon monoxide present in the float gas is, for obvious reasons, a better index.

Discussion

Illustrations of the histological nature of the gas-secreting pneumadena in *Physalia* are scanty indeed. Dahlgren and Kepner (1908) in their Figure 297 illustrated several secreting cells from *Physalia*. However, from my own observations it is evident that they confused the orientation of the ectodermal and gastrodermal layers in their sections and described instead the much longer columnar cells of the gastroderm with its characteristic brush border. This undoubtedly explains why Mackie (1960) was unable to identify the chromatic vacuoles of Dahlgren and Kepner in his sections of the pneumadena prepared by similar methods.

Okada (1935) presented drawings of the early developmental morphology of *Physalia* which depicted the gas gland as having tall columnar cells with basal nuclei and distal brush borders. His drawings were diagrammatic and were not intended to present accurate histological detail.

Mackie (1960) gave a description of the histology of the ectodermal layer of the pneumadena preserved from seven specimens. He described *columnar cells* as being in the majority with occasional *giant cells* scattered among them. In one specimen only he also saw clusters of *islet cells*. Mackie concluded that (p. 391) "The appearance of the cells in the gas gland varies markedly from one specimen to the next, and it is not clear to what extent this variability is due to differing ages of specimens, differing physiological states at the time of fixation or to differing methods of fixation." I have not been able to identify giant cells or islet cells in my own preparations and suspect that Mackie was examining tissues that were in poor condition before fixation.

Carbon monoxide is probably the only gas secreted by the pneumadena (Hahn and Copeland, 1966). Its survival value to the *Physalia* may exist in the fact that the solubility coefficient, and thereby the diffusivity, of the gas is approximately 30 times less than that of the less toxic carbon dioxide and therefore the carbon monoxide gas is more readily retained by the highly hydrated float tissue layers (Hahn and Copeland, 1966). There is no convincing evidence in the present studies that gas is released in the form of bubbles arising within the cytoplasm of the ectodermal cells. Since only one gas may be involved, it can be released readily by direct diffusion from the cell surface. This differs from the situation in the teleost swim bladder where accumulation of multiple molecular species of gases is dependent on physical phenomena associated with cytoplasmic microbubble formation by at least one of the involved gases (Wittenberg, 1958; Copeland, 1968).

The most striking specialization to be seen in the secretory cell is the peculiar

Figure 11. Material fixed in Gulf of Mexico and considered to be transitional between condition in Figures 9 and 10. "Gas release vesicles" similar to those in Figure 10 are seen. Mitochondria, though in poor condition compared with those shown in Figure 9, have not completely degenerated. 11,500 ×.

morphological organization of the mitochondria. The cristae are markedly reduced in size and number. Conversely, the matrix is tremendously hypertrophied in comparison with that in most types of mitochondria. It would be interesting to know if the respiratory enzymes of this peculiar mitochondrion are limited to the membranes. It may also be that the granular matrix in some way is associated with the high concentration of folates observed by Wittenberg, Noronha and Silverman (1962) which are thought to be the probable coenzymes involved in the production of the carbon monoxide gas.

The gas-secreting cell is almost devoid of the cytoplasmic morphology characteristic of most secretory cells. Smooth endoplasmic reticulum characteristic of steroid-producing cells is sparse and the rough variety associated with protein production is particularly elusive. The Golgi complexes are reasonably numerous and are well organized. However, the dark bodies or vesicles from the delivery side of the Golgi complex disappear without formation of anything resembling a secretory granule. The multivesicular bodies are numerous enough to suggest some functional role in the gas secretion. That role could well be one of degradation rather than synthesis, *i.e.*, the bodies may be lysosomal in nature. In short, the gas-secreting cell of the *Physalia* pneumadena is highly specialized for its peculiar function and it is difficult to find cytoplasmic homologies in other cell types.

There is no adequate explanation for the cisternal space, with its bounding membranes, frequently observed parallel to the nuclear membrane. It might in some way serve as a barrier between the nuclear contents and the general cytoplasm for an obscure reason. It may equally well be a rather unusual signal of pending degeneration in those particular cells.

In view of the markedly different observations of the Massachusetts forms as compared with the Mississippi Delta forms, I am reluctant to claim that those from the Delta area are completely normal and healthy animals. Presumably they moved from the Sargassum Sea area of the equatorial Atlantic Ocean on the primary Gulf Stream which swings north through the Yucatan Straits, then eastward to the tip of Florida and then up the Eastern Coast. A branch of this current comes almost straight north from the Yucatan Straits into the Mississippi Delta area *via* the old DeSoto Canyon. In terms of time-distance lapse, the Delta forms should be reasonably healthy, especially the smaller (younger) ones. A quick answer could be obtained by analyzing the gas of *Physalia* collected in their rearing grounds. If concentrations of carbon monoxide higher than 35% are observed, then the fine structure of the pneumadena would require further investigation.

There is one puzzling point not answered by the present study. The animals captured off Gay Head Light, Massachusetts, looked normal externally, with well inflated floats. Unless the permeability of the pneumatosaccus and pneumatocodon had been markedly reduced, the animals were still secreting some gas, albeit at a reduced rate, from tissue in which the mitochondrial membranes had disappeared. If it is assumed that the substrate-enzyme system for carbon monoxide production is associated with the granular matrix of the mitochondria, then that system might still be intact to some degree in the distal ends of the degenerating cells. In that event, the complex of vesicles seen in Figure 10 might bear in last analysis a

functional relationship to the modified system to produce a lowered level of gas release.

We are indebted to the Freeport Sulphur Company for the occasional use of their seaplane to locate the blue water interface which is usually in the form of a tide-rip. Its location varies tremendously with wind, tide and the general set of Gulf water movement (which is usually from east to west across the end of the Mississippi River Delta). We are also indebted to the Coast Guard for their most welcome help on two separate emergency occasions.

Summary

1. The carbon monoxide gas-secreting tissue (pneumadena) of *Physalia* is a single layer of ectodermal cells that are cuboidal to columnar in morphology. There is only one specific type cell and it is characterized by having the distal part, adjacent to the gas-interface surface, packed with large mitochondria. The mitochondria are unique in having few and small cristae. Most of the internum of the mitochondrion is occupied by a granular matrix. Compared with other secreting cells, there is very little smooth endoplasmic reticulum and less rough endoplasmic reticulum. There are numerous Golgi complexes and complex multivesiculate bodies. The nucleus is often enveloped by a cisternal space.

2. The secretory epithelium is sensitive to adverse factors such as lowered temperature and presents changes that, though degenerate in appearance, may still have some functional ability to produce carbon monoxide. There is no evidence to indicate conclusively that gas is secreted by formation of vesicles in the cytoplasm. Therefore, it is assumed that carbon monoxide is secreted by diffusion from the cell surface which is frequently thrown into finger-like projections.

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